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Cellular Immune Response of Ducks to Duck Hepatitis B Virus Infection

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Duck hepatitis B virus (DHBV) has been a useful model for hepadnavirus infection. There have been few studies on immunity to DHBV and none describing the cell-mediated immune response by acute and chronically infected ducks. A duck hepatitis B antigen-specific blastogenesis assay was used to measure DHBV antigen-specific responses of duck peripheral blood (PBMC) and splenic mononuclear cells (SMCs) from uninfected control ducks, ducks acutely or chronically infected with DHBV, and ducks immune to DHBV. A comparison of the group mean responses by PBMC to DHBV surface antigen (DHBsAg) found that the immune group was significantly different to the other three groups (controls or unexposed, $P < 0.0001$; acutely infected, $P < 0.01$; chronically infected, $P < 0.01$). The responses to DHBsAg by PBMC of the acute group ($P < 0.01$) were significantly different also to that of the unexposed group. For DHBV core antigen (DHBcAg), significant differences in the responses were found between immune ducks and unexposed ($P < 0.0005$) and acutely infected ($P < 0.05$) groups. The SMC showed a significant difference between unexposed ducks and immune ducks ($P < 0.05$) in the group mean responses to DHBsAg. The responses to DHBcAg were significantly different between the immune group and the acute ($P < 0.01$) and unexposed ($P < 0.01$) groups. The group mean of unexposed ducks was also significantly different to that of acutely infected ducks ($P < 0.01$). This study indicates that the cellular immune response in immune animals differs from acutely and chronically infected ducks. Further studies of these differences may provide some explanations for the differing outcomes of DHBV infection. ***J. Med. Virol. 58:19–25, 1999.***

KEY WORDS: hepadnavirus; cell-mediated immunity; antigen-specific blastogenesis

INTRODUCTION

Hepatitis B infection (HBV) and its outcomes are major health problems placing large demands on health resources. The clinical consequences of HBV infection are dependent on the interaction of host and viral factors, central to which is the specific host immune response. This immune response is considered to be the key element in the final outcome, namely, clearance or chronic persistence of the virus, and is the main cause of the observed cytopathology of the liver.

Despite evidence for the importance of cell-mediated immune (CMI) in the host response to HBV infection [Mondelli et al 1982; Pignatelli et al., 1987; Ferrari et al., 1990; Chisari, 1995; Penna et al., 1996; Rehermann et al., 1996], the exact mechanisms have not been fully determined, and, as a corollary, the reasons for the variable outcomes are not understood. As successive methods for demonstrating cell-mediated immune functions have been devised, they have been applied to the problem. Neither the ability to identify and separate different lymphocyte subclasses nor the availability of pure antigens have given clear results. This is mainly attributable to the difficulty of determining the disease status of human subjects, and the practical and ethical difficulties of studying CMI responses especially in early acute, uncomplicated hepatitis B infection.

Progress in the study of HBV has depended to a large extent on animal models. The duck/DHBV model [Summers and Mason, 1982] is especially valuable because the timing of infection and the quantity of the infectious dose produces different clinical outcomes. Experimental infection of ducks 1 to 3 days of age results in persistent infection with viremia [Omata et al., 1984; Fukada et al., 1987] similar to chronic HBsAg carriage occurring in neonates [Beasley et al., 1983]. Injection of older ducklings with DHBV intravenously results in a

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proportion becoming infected, the proportion dependent on the virus titer inoculated [Vickery and Cossart, 1996; Jilbert et al., 1998]. High titer inocula lead to a greater proportion of ducks becoming chronic carriers; a lower virus dose results in transient infection, with clearance of the virus from both the serum and the liver. This is analogous to acute HBV infection in adults.

The potential to manipulate the duck immune system, as well as the range of clinical syndromes possible with the DHBV/duck model, justifies the study of the role of the duck immune system in these infection outcomes. The study reported is the first time the cellular immune response to native DHBV surface (DHBsAg) and core (DHBcAg) antigens has been assessed in uninfected, acute or chronically infected, and immune ducks. One measure of CMI, an antigen-specific blastogenesis assay [Vickery et al., 1997], was used.

MATERIALS AND METHODS

Ducks

All ducks were Pekin-Aylesbury cross birds originating from a DHBV-negative commercial flock. Both male and female ducks were used. The use of animals in this experiment was approved by the University of Sydney's Animal Care and Ethics Committee.

Experimental Groups

Uninfected control ducks. These were 20 ducks not exposed to DHBV and were obtained from a DHBV-free flock. Their age ranged from 1 to 12 months.

Acutely infected ducks. These nine birds were inoculated with DHBV at 4 weeks of age. Two ducks received 10^6 ID_{50} doses (corresponding to 1,000 times the 50% infectious dose for 26-day-old ducks [Vickery and Cossart, 1996]), four ducks received 10^5 ID_{50}, and three ducks received 10^4 ID_{50} doses.

Chronically infected ducks. All five birds in this group were persistently viremic for at least 53 days after infection and were considered chronic carriers. Three ducks were infected with 10^5 ID_{50} DHBV at day 1 of age. These ducks were viremic by day 14 and remained persistently viremic, anti-DHBs antibody-negative, and anti-DHBc antibody-positive. Two ducks were infected with $\geq 10^5$ ID_{50} DHBV at 4 weeks of age also failed to clear the infection.

Immune ducks. There were two subgroups of ducks within the 13 of this group: one, immune following intraperitoneal vaccination with purified native envelope antigen (25-μg/kg body weight) on days 6, 12, and 26 posthatch (eight ducks); two, immune after being infected at 4 weeks and clearing the infection thereafter (five ducks).

All the immune ducks were challenged with 5 ml of DHBV-positive sera containing 1×10^9 virions/ml, approximately 1 week prior to CMI evaluation. Following challenge all ducks remained DHBV-negative in the sera by PCR and were DHBV-negative in the liver at euthanasia.

Immune Response of Ducks

Ducks were bled up to 12 times over a 12-week period to determine anti-DHBs and anti-DHBc antibody status. The antigen-specific responses of duck Peripheral blood mononuclear cells (PBMC) were tested as follows: control ducks were bled on only one occasion while immune and infected ducks were bled between one and four times. PBMC were separated and cultured as described below. The antigen-specific responses of duck splenic mononuclear cells (SMCs) were examined following euthanasia. SMC were obtained from 4 control, 12 immune, and 8 infected ducks. The SMCs were cultured as below.

Assessment of DHBV Status

Experimental ducks were bled when 1-day-old and at intervals similar to those for detection of antibody. The serum samples were tested for DHBV DNA by dot-blot hybridization as described by Freiman et al. [1988]. At the end of the experiment, 200 mg of liver from each bird were used to determine the DHBV DNA status by dot-blot hybridization as described by Freiman et al. [1988].

PCR for a 340-bp segment of the precore region of DHBV was performed on serum samples that were negative on dot-blot hybridization as described by Vickery and Cossart [1996]. The product specificity was checked by either DNA sequencing or by an independent PCR of the surface region.

Viral Antigens

Viral antigens used for the antigen-specific blastogenesis assay were prepared as described by Vickery et al. [1997]. DHBsAg was purified from high titer DHBV-infected serum and DHBcAg from infected livers by the procedures of Summers and Mason [1982] and Marion et al. [1983]. The purity of both antigens was confirmed by Western blot analysis using rabbit anti-DHBsAg antibody [Vickery et al., 1989] and rabbit anti-DHBcAg antibody raised using *E. coli*-expressed DHBcAg (a kind gift from Dr. Allison Jilbert, University of Adelaide) [Jilbert et al., 1992].

Detection of Anti-DHBs and Anti-DHBc Antibody

Antibodies against DHBsAg and DHBcAg were detected by indirect ELISAs with sera diluted 1:4 as described by Vickery and Cossart [1996]. The results for both assays were calculated from the percentage inhibition of the respective rabbit antiserum. Antibody activity was defined as a percentage inhibition greater than two standard deviations from the mean value calculated for ducks unexposed to DHBV. The cutoff for anti-DHBs activity was an inhibition of >12 ($n = 40$ negative controls) and for anti-DHBc activity was an inhibition of >31 ($n = 17$ negative controls) [Vickery et al., 1989].

Separation of Peripheral Blood and Spleen Mononuclear Cells

Peripheral blood mononuclear cells were collected from 7 ml of blood by separating over Ficoll-Paque (Pharmacia, Uppsala, Sweden) as described by Vickery et al. [1997]. Spleen mononuclear cells (SMC) were separated over Ficoll-Paque by the method of Vickery et al. [1995].

Antigen-Specific Blastogenesis Assay

The optimization of this assay was described by Vickery et al. [1997]. The separated PBMC and SMC were counted and their viability assessed by trypan blue exclusion. Cells were cultured in 96-well flat-bottom microculture plates (Nunc) in 200-μl RMPI 1640 with 20-mM Hepes and 23-mM $NaHCO_3$, 100-IU benzylpenicillin, 100-μg dihydrostreptomycin sulfate/ml, and supplemented with heat inactivated 10% pooled duck serum (PDS). Plates were incubated at 41°C in a humidified 5% CO_2 in air atmosphere.

Mitogen or antigen was added at the same time the cultures were established. PHA (10 μg/ml) was added to cells cultured in three wells of each microculture plate to monitor whether culture conditions were suitable for blastogenesis and whether each batch of cells would respond to mitogenesis. DHBsAg was added at two concentrations: 0.01 μg/ml and 0.1 μg/ml. As only a limited amount of native DHBcAg was available, only one concentration of 0.1 μg/ml was used.

It was shown that optimum cell numbers/well for antigenic blastogenesis could be predicted by a prior determination of the individual animal lymphocyte response to PHA [Vickery et al., 1997]. Each duck was bled once and its PBMC cultured in PHA (10 μg/ml) at different cell numbers/well. For most ducks, this optimum was 8×10^5 cells/well.

Each variable was tested in triplicate. Cells were cultured from day 4 to day 8 inclusive before being radiolabeled for 6 hr by the addition of 20 μl of RMPI 1640 containing 1 μCi of ^{3}H thymidine (ICN 2406705, Irvine) and harvested onto glass-fiber mats (GF/C, Whatman, Maidstone). The ^{3}H thymidine incorporation was read in an LKB 1214 Rakbeta Counter.

All cultures included unstimulated labeled and unstimulated unlabeled controls. The PBMC and SMC responses to antigens were measured by ^{3}H uptake (dpm). The stimulation index (SI) was calculated using the mean dpm by the formula:

$$\frac{(\text{stimulated } ^3\text{H-labelled})\text{-}(\text{unstimulated unlabelled})}{(\text{unstimulated } ^3\text{H-labelled})\text{-}(\text{unstimulated unlabelled})}.$$

A positive SI was a response to antigen stimulation that was significantly different from the unstimulated controls as determined by Student's *t*-test. For ducks that were bled more than once, the maximum significant SI value obtained was used for analysis.

Liver Histopathology

Liver for histopathology was removed after the ducks were euthanased and was fixed in 10% neutral buffered formalin for 48 hr. The tissue was paraffin-embedded and stained with hematoxylin and eosin using standard techniques.

Livers were examined histologically and given a score based on the presence and degree of inflammation: normal liver (score 0), no inflammatory cells; slight inflammation (score 1), occasional foci of inflammatory cells, especially along portal tracts; mild inflammation (score 2), foci of inflammatory cells along most portal tracts with some bile duct proliferation; moderate inflammation (score 3), inflammatory cells present in parenchyma along septa with or without piecemeal necrosis; and severe inflammation (score 4), inflammation accompanied by either regenerative nodules, extensive septa formation, or areas of necrosis and collapse.

Statistical Analysis

Student's *t*-test was used to examine significant differences in blastogenesis transformation between stimulated cultures and control cultures for each individual duck. The Mann-Whitney U-test was used to test for significant differences between the group mean responses. Differences in liver histopathology scores between groups and between ducks of different DHBV status were assessed by the Wilcoxon's rank sum test.

RESULTS

The results for antibody and virus status, and the antigen-specific blastogenesis assay for the four groups of ducks are summarized in Tables I and II. Individual duck results for antigen-specific blastogenesis of PBMC are shown in Figures 1 and 2.

Uninfected control ducks (n = 20). None of these had DHBV DNA or anti-DHBs antibody. Only one duck reacted to the anti-DHBc assay at a low level. There was little response to DHBsAg or DHBcAg by PBMC or SMC from these ducks.

Acutely infected ducks (n = 9). Three of the infected ducks cleared the infection completely, three cleared DHBV from the serum but not the liver, and the remaining three became persistently viremic (Table I). The humoral element of the host response related to the outcome of infection. All ducks developed anti-DHBc antibody usually within 1 week of infection. Anti-DHBs production was only evident when ducks were nonviremic.

The response by PBMC from acutely infected ducks in the blastogenesis assay varied over time. Five of the nine ducks showed a response to DHBsAg and two to DHBcAg within 10 days of infection. Between 4 and 7 weeks postinfection, PBMC from a further three ducks responded to DHBsAg and from another two birds to DHBcAg.

SMC were tested from two acutely infected ducks at 17 and 38 days postinfection. These ducks were DHBV-

TABLE I. Group Mean Stimulation Indexes for Peripheral Blood Mononuclear Cells Exposed to Duck Hepatitis B Virus Antigens in an Antigen-Specific Blastogenesis Assay, DHBV Status in Liver and Serum, and Antibody Status to DHBcAg and DHBsAg in Uninfected Control, Acutely Infected, Chronically Infected, and Immune Ducks[a]

Duck group	Number of ducks	DHBsAg (SI ± SE)	DHBcAg (SI ± SE)	DHBV DNA			Anti-DHBs	Anti-DHBc
				Serum initial[b]	Serum end[c]	Liver		
Uninfected control	20	1.50 ± 0.29	0.94 ± 0.25	0	0	0	0	1
Acutely infected	3			2	3	3	2	3
	3	3.66 ± 0.72	1.49 ± 0.53	3	0	3	3	3
	3			0	0	0	3	3
Chronically infected	5	2.39 ± 0.92	2.02 ± 0.79	5	5	5	0	5
Immune	13	8.74 ± 1.30	3.26 ± 0.50	0	0	0	13	13

[a]DHBV: duck hepatitis B virus; SE: standard error; DHBsAg: duck hepatitis B surface antigen; DHBcAg: duck hepatitis B core antigen; anti-DHBs: antiduck hepatitis B surface antigen antibody; and anti-DHBc: antiduck hepatitis B core antigen antibody.
[b]DHBV status of the serum of ducks at the beginning of experiment.
[c]DHBV status of the serum of ducks at the end of experiment.

TABLE II. Group Mean Stimulation Indexes for Splenic Mononuclear Cells Exposed to Duck Hepatitis B Virus Antigens in an Antigen-Specific Blastogenesis Assay, DHBV Status in Liver and Serum, and Antibody Status to DHBcAg and DHBsAg in Uninfected Control, Acutely Infected, Chronically Infected, and Immune Ducks[a]

Duck group	Number of ducks	DHBsAg (SI ± SE)	DHBcAg (SI ± SE)	DHBV DNA			Anti-DHBs	Anti-DHBc
				Serum initial[b]	Serum end[c]	Liver		
Uninfected control	4	0.99 ± 0.02	0.92 ± 0.01	0	0	0	0	0
Acutely infected	2	1.75 ± 0.75	−0.19 ± 0.19	2	0	2	2	2
Chronically infected	5	2.29 ± 0.78	1.62 ± 0.94	5	5	5	0	5
Immune	12	4.67 ± 1.53	2.27 ± 0.62	0	0	0	12	12

[a]DHBV: duck hepatitis B virus; SE: standard error; DHBsAg: duck hepatitis B surface antigen; DHBcAg: duck hepatitis B core antigen; anti-DHBs: antiduck hepatitis B surface antigen antibody; and anti-DHBc: antiduck hepatitis B core antigen antibody.
[b]DHBV status of the serum of ducks at the beginning of experiment.
[c]DHBV status of the serum of ducks at the end of experiment.

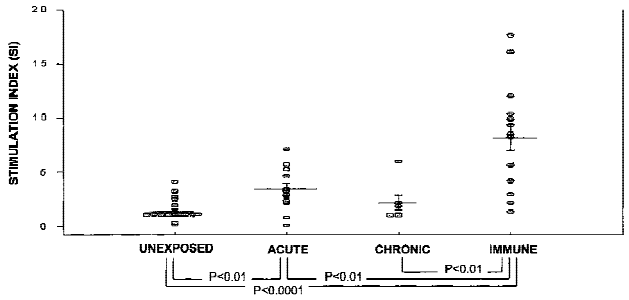

Fig. 1. Transformation responses as measured by the stimulation indexes (SI) of duck peripheral blood mononuclear cells to DHBsAg in unexposed control, acutely infected, chronically infected, and immune ducks. Statistically significant differences between groups are indicated.

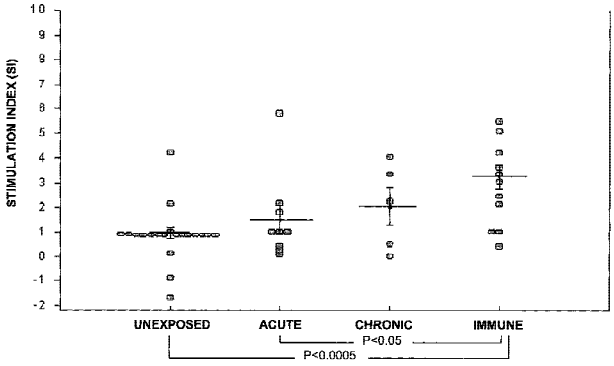

Fig. 2. Transformation responses as measured by the stimulation indexes (SI) of duck peripheral blood mononuclear cells to DHBcAg in unexposed control, acutely infected, chronically infected, and immune ducks. Statistically significant differences between groups are indicated.

positive in the liver despite clearing the infection from the circulation and developing anti-DHBs and anti-DHBc antibodies (Table II). A positive response to DHBsAg ($P < 0.01$) was demonstrated in one duck.

Chronically infected ducks (n = 5). These chronic carriers had persistent viremia and were positive for anti-DHBc antibody. Anti-DHBs antibody was not detected at any time (Table I).

PBMC from chronically infected ducks showed varying SI values demonstrating the variable nature of the immune response in chronically infected ducks (Figs. 1 and 2). PBMC from three of five ducks were significantly stimulated by DHBsAg and DHBcAg (P varied between 0.05 and 0.01). There was no difference in response to viral antigens between ducks infected at 1 day of age or those infected at 4 weeks of age.

There was significant stimulation of SMC by DHBsAg from three of five chronically infected ducks ($P < 0.01$). The transformation responses of SMC from one duck were significantly stimulated with DHBcAg ($P < 0.05$).

Immune ducks (n = 13). These remained negative by PCR for DHBV in sera pre- and postchallenge and

TABLE III. Histopathological Evaluation for Inflammation and DHBV Status of Livers From Ducks Infected or Not Infected With DHBV

Histopathology score	Number of ducks never infected	Number of ducks infected	
		Positive for liver DHBV DNA	Negative for liver DHBV DNA
Normal (score 0)	9	0	3
Slight inflammation (score 1)	1	2	0
Mild inflammation (score 2)	0	2	2
Moderate inflammation (score 3)	0	2	1

were negative in the liver at euthanasia. Following challenge, ducks had both anti-DHBs and anti-DHBc antibodies (Table I).

PBMC from all ducks in the immune group after challenge demonstrated significant positive transformation responses to stimulation by DHBsAg and from 10 of 13 birds to DHBcAg (P varied from 0.05 to 0.001) (Figs. 1 and 2). There were no differences in the transformation responses between ducks that had been vaccinated and challenged, or immune ducks challenged after past infection. However, the responses of PBMC fell to insignificant levels when four ducks were tested up to 4 weeks postchallenge (results not shown).

There was a lower number of significant stimulatory responses by SMC from immune ducks with 8 of 12 ducks showing significant stimulation to DHBsAg (P varied between 0.05 and 0.01). This was probably due to the harvesting of SMC up to 4 weeks postchallenge in contrast to PBMC, which were harvested from these ducks approximately 1 week postchallenge. The transformation response to DHBcAg by SMC from six ducks was significantly stimulated (P varied between 0.05 and 0.001).

Comparison of Group Cell-Mediated Immune Responses

The group mean responses are shown in Tables I and II.

PBMC blastogenesis. Analysis of the group mean responses by PBMC to DHBsAg (Fig. 1) found that the immune group was significantly different to the other three groups in the analysis (negative controls, $P < 0.0001$; acutely infected, $P < 0.01$; chronically infected, $P < 0.01$). The responses to DHBsAg by PBMC of the acute ($P < 0.01$) were significantly different to that of the unexposed group. No significant differences were found between the acutely and chronically infected or the unexposed and chronically infected groups.

For DHBcAg, significant differences in the responses were found only between immune ducks and unexposed ($P < 0.0005$) and acutely infected ($P < 0.05$) groups (Fig. 2). There were no other significant differences between groups.

SMC blastogenesis. A comparison of the group mean responses of SMC to DHBsAg found that there was a significant difference between immune ducks and unexposed ducks ($P < 0.05$). There was no statistically significant difference between unexposed and acute or chronic groups.

There was a significant difference in the responses to DHBcAg by SMC between the immune and the acute ($P < 0.05$) and the immune and unexposed ($P < 0.01$) groups. The group mean of unexposed ducks was also significantly different to that of acute ducks ($P < 0.01$).

Histopathological Evaluation of Livers

Analysis of group differences in histopathology scores showed that the control group (n = 10) had significantly lower scores than the acutely and chronically infected groups (n = 11) ($P < 0.01$). The immune group (n = 9) had significantly lower scores than the combined acute and chronically infected groups ($P < 0.01$). No significant differences were observed between chronically infected (n = 4) and acutely infected (n = 7) groups.

Comparison was made between ducks that had been infected (n = 16) and those never infected (n = 14) (Table III). The difference in the degree of inflammation between these two groups was statistically significant ($P < 0.01$). Ducks still infected at the end of the experiment (n = 10) had significantly more hepatic inflammation than ducks that had never been infected or had cleared DHBV (n = 20; $P < 0.01$). There was no significant difference in hepatic inflammation between ducks that had been infected transiently clearing the virus from their livers and those that were still DHBV-positive. This may be due to the small numbers of animals involved, as three of six animals that had cleared DHBV had normal histology while all 10 DHBV-positive ducks had abnormal histology (Table III).

DISCUSSION

This study has shown that there is a CMI response by ducks to DHBV and that the response in immune ducks differs from uninfected, acutely or chronically infected birds. The advantage of this duck model is that it can generate a variety of infection outcomes resulting in acute or chronic infection. The duck immune system can be manipulated through the bursectomy or thymectomy of ducklings, and the cellular immune response in blood and liver can be monitored in conjunction with the detection of virus. Therefore, the duck model has potential in examining the immune response to hepadnaviruses in general, and to explain the variable outcomes of hepadnaviral infection.

In vitro antigen-specific blastogenesis exhibited by PBMC from the DHBV immune group to DHBsAg was significantly different from uninfected ($P < 0.0001$),

acutely infected ($P < 0.01$), and chronically infected ($P < 0.01$) groups. The responses to DHBsAg by PBMC of the acute ($P < 0.01$) group were also significantly different to that of the uninfected group. Although the transformation response of PBMC from immune ducks to DHBcAg was significantly different from uninfected ($P < 0.0005$) and acute ($P < 0.05$) ducks, the differences were of smaller magnitude than that obtained with DHBsAg.

In acute self-limiting HBV infection, the peripheral T-cell response to HBcAg and HBeAg is vigorous, with most patients exhibiting an SI > 10 [Ferrari et al., 1990; Jung et al., 1991], and is related temporally to HBsAg clearance [Ferrari et al., 1990]. The magnitude of the transformation response to nucleocapsid antigens is greater than the response elicited by envelope antigens in both acute [Ferrari et al., 1990; Jung et al., 1991] and chronic [Sylvan et al., 1987; Ferrari et al., 1990] HBV infection, suggesting a major role for nucleocapsid antigens in the clearance of HBV. Over the course of this experiment, PBMC from only three of nine ducks with acute DHBV responded to DHBcAg despite all developing anti-DHBc antibodies, while eight of nine responded to DHBsAg. However, two of the three ducks responding to DHBcAg cleared DHBV completely and the other cleared it from the circulation. The lower response of duck PBMC to DHBcAg when compared with DHBsAg may only be a reflection of the assay used as the antibody responses to DHBV and HBV appear to be similar. However, other researchers have suggested a similar major role for surface antigen in HBV infection and clearance [Tong, 1975; Barnaba et al., 1989; Nagafuchi et al., 1989]. Seven of the eight ducks responding to DHBsAg either temporarily or permanently cleared DHBV from the circulation (two cleared the infection completely). In the duck, as in humans, it is still not clear which viral antigen is the most important as regards immunopathogenesis of disease, viral clearance, or persistence. This might be clarified if recombinant DHBcAg at different concentrations were used in the blastogenesis assay.

Five chronically infected birds were tested. In this study, chronic infection was defined as DHBV DNA-positive in the serum more than 53 days postinoculation. This definition was based on the observation of Jilbert et al. [1992] that viremia in adult ducks was fleeting and DHBV clearance from the liver was generally achieved by 40 days postinoculation in transient or acute infection. Although every bird developed anti-DHBc antibody, only three of five had PBMC that demonstrated core antigen-specific transformation. Similarly, in human chronic hepatitis B, transformation responses to HBcAg were only detected in approximately 50% [Ferrari et al., 1986, 1990] and 20% [Jung et al., 1991] of chronically infected patients. Anti-DHBs activity was not detected in any of the chronic infected ducks; however, positive transformation to DHBsAg was detected in three of five ducks. In human chronic HBV infection, some researchers have been unable to detect lymphocyte transformation to HBsAg [Hanson et al., 1984; Ferrari et al., 1986]. Others have detected transformation in some patients, especially those with elevated liver enzymes [Beutner et al., 1978; Tiku et al., 1978; Jung et al., 1991], suggesting a role for DHBsAg in the pathogenesis of HBV infection.

Ferrari et al. [1990] and Jung et al. [1991] have suggested that antigen-specific T-cells may be concentrated at the site of liver injury rather than in the peripheral circulation. Core antigen-specific T-cells with helper and suppressor activities [Ferrari et al., 1987] and PreS2-specific helper and cytotoxic T-cells have been found in the hepatic mononuclear cell infiltrate [Barnaba et al., 1989]. The loss of antigen-specific cells to the liver may also occur in the duck. In this study, 5 of the 14 infected ducks showed significant inhibition to DHBcAg and 1 to DHBsAg. In contrast, only 1 of 13 of the immune ducks showed any inhibition and that was to DHBcAg. Alternatively, hepadnaviruses may be able to affect MHC expression, thus influencing the immune response (reviewed by McFadden and Kane [1994]). The substantial number of occasions in which in vitro suppression could be demonstrated in DHBV-infected animals suggests that viral-specific immune suppression may play a crucial role in the pathogenesis of DHBV infection [McFadden and Kane, 1994; Kotwal, 1997].

Ducks that became immune following transient infection with DHBV or following vaccination demonstrated PBMC antigen-specific transformation to both antigens after challenge with DHBV. However, this CMI response is temporary and in some ducks fell to nonsignificant levels quickly. This needs further investigation in a long-term study.

The unavailability of lymphocyte markers for the duck prevented the manipulation of accessory cell numbers for a standard monocyte:lymphocyte ratio in culture. This may have caused poor transformation responses in some of the ducks. However, Ferrari et al. [1989] found lymphocyte transformation to stimulation with either HBcAg or HBsAg equal in unfractionated PBMC or in purified T-cell cultures with autologous mitomycin C-treated non–T-cells.

A part of this study was the histopathology of livers from the different groups of ducks. Ducks infected with DHBV had more severe hepatic inflammation than ducks that had never been infected either by being controls or by being immune. This was true irrespective of whether or not they cleared the virus (Table III), although half of the ducks that had cleared DHBV from the liver showed normal liver histology. This current study demonstrates the importance of infection in the development of hepatic inflammation. Recently, Bertram et al. [1996] have shown that polyclonal CD3 detects duck T-cells. Further determination of a range of lymphocyte markers for the duck will enable the identification of what mononuclear cells infiltrate DHBV infected livers.

The refinement of the antigen- specific blastogenesis assay, the utilization of lymphocyte markers and ge-

netically engineered antigens, and the identification of epitopes responsible for immune recognition of DHBV will allow further exploitation of the duck model. Other measures of cell-mediated immunity will also need to be developed. Applying this knowledge to the in vivo model will lead to an understanding of how the specific elements of immune defense complement each other in viral removal.

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